Scientific Company (Pittsburgh, PA). Absolute ethyl alcohol was obtained from Warner-Graham Company (Cockeysville, MD), and Tris was obtained from Schwarz/Mann Biotech (Cleveland, OH).

Whole mammary glands from three midlactation, multiparous Holstein cows of known good health and productivity ($\bar{X}$: 19.5 kg of milk/d) were obtained from the herd at USDA, Beltsville, MD. Whole mammary glands were obtained at slaughter, trimmed to remove adipose tissue, and sectioned into pieces approximately 10 × 15 × 5 cm. Rat mammary tissue was pooled from three Sprague-Dawley females, 9 to 12 d postpartum (West Jersey Biological, Wenovah, NJ). The tissue was either processed immediately or frozen on dry ice and stored at –80°C until used. Tissue fractionation was as previously described in detail by Basch et al. (1).

Enzyme Assays

The OAT was measured by the method of Jenkins and Tsai (8). Each enzyme assay contained 25 m*M* ornithine, 7.5 m*M* sodium pyrophosphate, 8.75 m*M* α-ketoglutaric acid, and .2 mg/ml of pyridoxal-5-phosphate in a 250-μl volume. For studies of the subcellular distribution, OAT assays were incubated at pH 8.0 and at 37°C for 30 min with 50 μl of protein (cytosol at protein concentration of 6 mg/ml or mitochondria at 4 mg/ml) and had zero time blanks. The blanks were used to correct for any nonspecific activity. At the end of the incubation, 250 μl of cold trichloroacetic acid were added to the reaction mixture to stop the reaction. Next, 500 μl of cold, filtered *o*-aminobenzaldehyde (.5% in 95% ethyl alcohol) were added into the reaction mixture to form a stable complex, and the color was allowed to develop at 37°C for 10 min. The mixture was then centrifuged at 10,800 × *g* for 15 min at 25°C with a TOMY microcentrifuge (Peninsula Laboratories, Inc., Belmont, CA). The supernatant was carefully pipetted and transferred into a cuvette, and the optical density at 440 nm was measured with a Gilford spectrophotometer (Gilford Instruments, Oberlin, OH). All assays were carried out within the linear range of the reactions with respect to incubation time and enzyme concentration, had a total volume of 1.0 ml, and had an incubation temperature of 37°C.

The activities of citrate synthase were measured spectrophotometrically according to the procedure with the rat liver as described by Shepherd and Garland (15). The reaction mixture in disposable 1-cm polystyrene cuvettes contained 2.0 ml of .1 *M* Tris buffer (pH 8.0), 20 μl of 10 m*M* DTNB, 20 μl of 50 m*M* oxalacetate (pH 7.5), 10 μl of 5 m*M* acetyl-CoA, and 50 μl of the bovine mammary gland fraction containing the enzyme. The assay was performed at 25°C. Readings were recorded at 412 nm with a Gilford recording instrument that was attached to the Beckman spectrophotometer (Beckman Instruments, Fullerton, CA). The reaction was started by the addition of the enzyme. A blank contained all components except the mammary fraction studied. The amount of enzyme used was adjusted so that the slope, or the rate of increase in optical density over a period of time, was about 45° greater than that of the baseline.

One unit of enzyme is defined as the amount of protein required to synthesize 1 μmol of citrate/min. Specific activity is expressed as units per milligram of protein.

The methods mentioned by Basch et al. (1) were used to assay NADPH-cytochrome *c* reductase and succinic dehydrogenase.

Protein Assay

Protein was determined using the Pierce BCA (bicinchoninic acid) protein assay reagent (Pierce Chemical Company, Rockford, IL) with the room temperature protocol. Bovine serum albumin was the standard.

RESULTS

Reaction Conditions

Preliminary studies on lactating bovine mammary gland showed OAT activity in both cytosolic and mitochondrial fractions. The accuracy of the enzymatic assay was tested on both fractions. The enzyme assay was found to be linear with time for up to 50 min for both fractions. A linear response was found for up to 70 μl of cytosolic and 50 μl of mitochondrial preparations. Thus, the chosen assay (8) appears to work quite well in mammary fractions.

Having established the conditions for linearity, both fractions were assayed to determine

the optimal pH. Both bovine mammary cytosolic and mitochondrial fractions gave very sharp pH maxima at 8.0 (Figure 1), in contrast to the pH optima of 7.5 displayed by rat tissue preparations (7, 16).

Subcellular Distribution

Three subcellular preparations were made from frozen mammary tissues of three cows. The fraction of OAT activity in the cytosol appears to be considerable (Table 1), but the specific activity ratio of the subcellular fraction to homogenate is greatest for mitochondria. If, indeed, OAT is a mitochondrial enzyme, this result is puzzling because the ratio of succinate dehydrogenase in the mitochondrial fraction to that of homogenate was 5.8 ± .5 to 1 as expected (1). Because OAT is considered to be a mitochondrial matrix enzyme, the 56% (mean of four trials) in the cytosol could reflect mitochondrial breakage during freezing and thawing of the tissue or mitochondrial disruption during mincing and homogenization of the tissue. To test the effect of freezing and thawing or tissue damage, mammary tissue from three rats was pooled and treated in a similar fashion. (Fresh mammary tissue was immediately excised after rats were killed in the laboratory.) The subcellular distribution of OAT in the rat mammary tissue

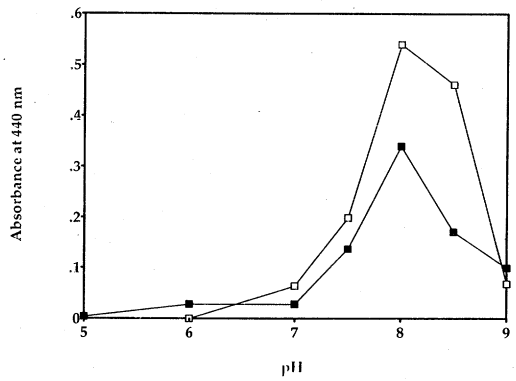

Figure 1. pH activity curves for ornithine aminotransferase of mitochondrial (□) and cytosolic (■) fractions of bovine mammary gland.

is also given in Table 1. Here again, although the specific activity is greater in the mitochondrial fraction, the yield of OAT in cytosol is nearly equal to that of the mitochondrial fraction.

To test further that the cytosolic OAT may be due to mitochondrial breakage, citrate synthase was also assayed in all fractions. The results for citrate synthase were similar to those obtained for OAT (Table 2). A similar distribution of activity was found for citrate

TABLE 1. Subcellular distribution of ornithine aminotransferase (OAT) in preparations from lactating mammary glands.

Fraction	Specific activity (nmol/min per mg of protein)		Yield (%)		Ratio to homogenate
	$\bar{X}$	SD	$\bar{X}$	SD	
Bovine[1]					
Homogenate	17.6	3.7	. . .	. . .	. . .
Nuclear	20.1	1.2	6	1	1.1
Mitochondrial	46.5	1.7	28	6	2.6
Microsomal	2.0	.4	1	1	.1
Cytosolic	19.0	6.1	56	10	1.1
Rat[2]					
Homogenate	4.8	. . .	. . .	. . .	. . .
Nuclear	13.7	. . .	2	. . .	2.9
Mitochondrial	18.3	. . .	40	. . .	3.8
Microsomal	.6	. . .	1	. . .	.1
Cytosolic	3.0	. . .	34	. . .	1.6

[1]Means and standard deviation from three preparations from three cows. Three enzyme assays were conducted for each preparation.

[2]Mean of one preparation from pooled tissue of three rats, assayed in triplicate.

TABLE 2. Subcellular distribution of citrate synthase in preparations of lactating mammary glands.

Fraction	Specific activity		Yield		Ratio to homogenate
	(nmol/min per mg of protein)		— (%) —		
	$\bar{X}$	SD	$\bar{X}$	SD	
Bovine[1]					
Homogenate	111	24	. . .	. . .	. . .
Mitochondrial	230	6	23	9	2.2
Cytosolic	137	34	64	6	1.2
Rat[2]					
Homogenate	282	. . .	. . .	. . .	. . .
Mitochondrial	967	. . .	43	. . .	3.4
Cytosolic	204	. . .	37	. . .	.7

[1]Means and standard deviation from three preparations from three cows. Three enzyme assays were conducted for each preparation.

[2]Mean of one preparation from pooled tissue of three rats, assayed in triplicate.

synthase from fresh rat mammary tissue. Thus, upon homogenization, both rat and bovine mammary glands show 60 to 40% breakage of the mitochondrial fraction, even though succinate dehydrogenase is primarily retained in the pellet fraction.

Heat Stability

Although current data (13) tend to indicate that there is only one gene for OAT and that it is a mitochondrial matrix protein, earlier literature argued for specific isozymes in selected tissues. Several groups (7, 11, 16) showed that subcellular preparations of homogenates and even purified preparations gave very different tissue-specific thermal and kinetic properties. Specifically, differences were observed for liver and kidney enzymes.

The thermal stability of the bovine mitochondrial and cytosolic fractions were tested and compared with published data for rat liver and kidney. Here the thermal stability was tested at selected temperatures with the standard assay (Figure 2). The two bovine preparations are similar to each other but different from the liver and kidney data. By applying the Arrhenius equation:

$$\log k = \frac{-E_a}{2.3R} \times \frac{1}{T} + C \qquad [1]$$

the activation energy (E_a) can be calculated from the left side of Figure 2, and the energy of activation for inactivation (E_{ai}) can be calculated from the right side. The apparent values are compared in Table 3. Clearly, the two mammary preparations are similar but different from the rat enzyme data taken from Volpe et al. (16) with respect to energy of activation for inactivation. With respect to activation energy, the rat kidney and bovine enzymes are probably the same, but rat liver has a much lower activation energy as can be seen by comparison of the velocity data of Figure 2.

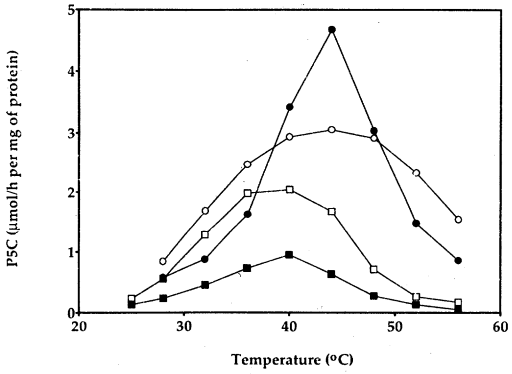

Figure 2. Comparison of the thermal activity curves for bovine mammary ornithine aminotransferase (□ = mitochondrial; ■ = cytosol) with published data (16) for rat liver (○) and kidney (●). Here reactions were under standard conditions but at the temperatures indicated. P5C = L-Δ^1-pyrroline-5-carboxylate.

TABLE 3. Apparent activation energy (E_a) and energy of activation for inactivation (E_{ai}) of ornithine aminotransferase activity of bovine mammary gland compared with values derived from published data (16) for rat liver and kidney.

Tissue	E_a	E_{ai}		
		(kcal)		
	$\bar{X}$	SE	$\bar{X}$	SE
Bovine mammary				
Cytosol	27.6	2.1	40.4	3.6
Mitochondria	35.2	4.7	42.8	1.8
Rat				
Liver	19.4	3.8	11.6	3.0
Kidney	27.8	2.6	29.8	2.1

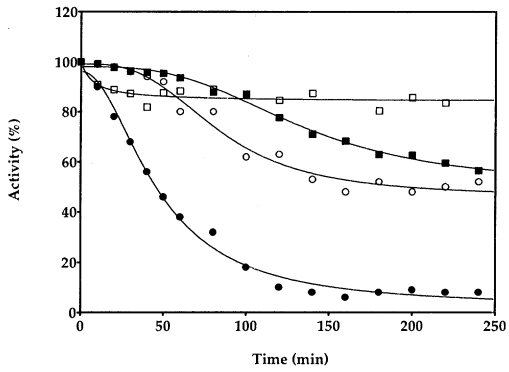

Figure 3. Comparison of the time-temperature reactivity of bovine mammary ornithine aminotransferase (□ = mitochondrial; ■ = cytosol) with published data (16) for rat liver (○) and kidney (●). Here enzymes were preincubated at 37°C at different times and then assayed under standard conditions. Half-lives and estimated maximum denaturation were calculated by nonlinear regression analysis as previously described (5).

A second thermal test is stability at 37°C against time. Here the bovine enzyme preparations differ somewhat from each other but clearly retain catalytic activity for longer incubation times than the rat liver and kidney enzymes (Figure 3). The half-life and estimated maximum percentage of activity were calculated by nonlinear regression analysis (5) and are given in Table 4.

Michaelis Constants

As just noted, rat liver and kidney enzymes differ considerably in heat stability but also in Michaelis constants (K_m). Here the mammary enzyme gave K_m values of 8.4 ± 1.0 and 17.9 ± 2.6 m*M* (Figure 4) for the mitochondrial and cytosolic fractions, respectively. These values were computed from direct nonlinear fits to velocity versus substrate concentrations (e.g., Figure 4) as previously described (5). The bovine mammary K_m values are both much greater than the 2 and 4 m*M* reported for OAT for rat liver and kidney, respectively.

DISCUSSION

Ornithine aminotransferase is considered to be a mitochondrial matrix enzyme that shuttles carbon skeletons and functions in the production of proline (7, 11, 16) in several tissue types. Studies on the uptake of proline by mammary cells have indicated that insufficient proline is taken up to support casein production by fully lactating cells (4, 10, 14), which would indicate a need for proline biosynthesis within the mammary gland (4, 10, 14). One study (14) using ^{14}C-labeled tracers indicated that ornithine could serve as a source of proline. This result is in line with earlier studies (4, 10) that showed that ornithine is readily taken up by lactating mammary cells. The mechanism for conversion of arginine or ornithine to proline could occur in two ways, but, in either case, no comparative studies of the occurrence of or subcellular localization of the enzymes in the mammary gland responsible for this conversion were conducted. A major first step in the conversion of ornithine to proline is the action of OAT to produce the δ-amino semialdehyde that cyclizes to yield L-Δ^1-pyrroline-5-carboxylate, which is then reduced by another enzyme to form proline.

TABLE 4. Comparison of thermal stability of bovine mammary enzyme preparations with values derived from published data for rat liver and kidney preparations (16).

Fraction	Half-life	Maximum denatured
	(min)	(%)
Bovine mammary		
Mitochondrial	10	20
Cytosolic	175	40
Rat		
Liver	60	50
Kidney	40	95

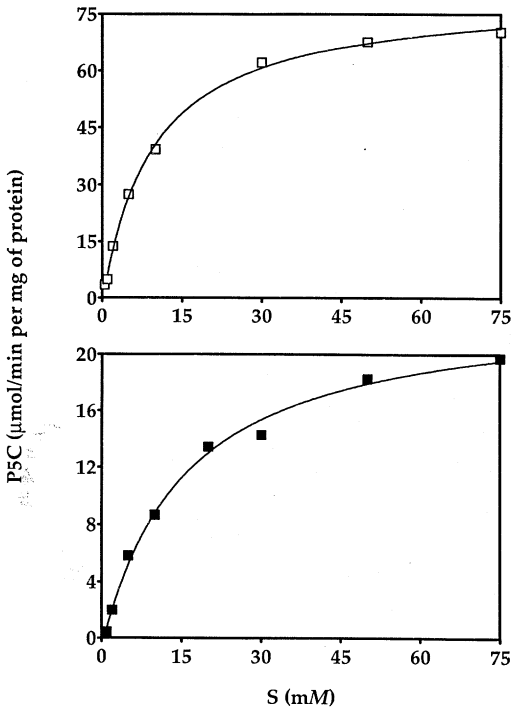

Figure 4. Change in the velocity of reaction of ornithine aminotransferase of bovine mammary mitochondrial (□) and cytosolic (■) fractions with increasing concentrations of ornithine (S). The Michaelis constants (K_m) of 8.4 and 17.9 m*M* were means of two complete experiments and were calculated by nonlinear regression analysis of data as previously described (5). P5C = L-Δ^1-pyrroline-5-carboxylate.

Results from our work show that the OAT enzyme is present in lactating mammary gland and that the specific activity in the crude homogenate is 3.7 times greater in bovine than in rat and the specific activity is twofold higher in the bovine mitochondrial fraction. There, apparently, is considerable breakage of the mitochondrial fractions of both species, as evidenced by parallel occurrence of citrate synthase and OAT in the cytosolic fractions.

Comparisons of the data (16) cited to support tissue specific isozymes for OAT predict divergent enzymatic properties for liver and kidney enzymes, but cDNA sequencing predicts identical polypeptide chains for rat liver and kidney enzymes (13). Several factors, such as posttranslational events (e.g., phosphorylation, lipid acylation, or proteolytic processing) or defects in mitochondrial transport (12), could yield differentially active enzymes. Hence, a priori, it was difficult to determine which enzymatic pattern mammary OAT would follow. Finally, Volpe et al. (16) presented data to indicate that cell cycle stage could alter the type of OAT isozyme present. In this respect, one could predict behavior similar to that in kidney that is associated with G_1 (growth) phase (16); Keys et al. (9) have recently shown by flow cytometry that >90% of bovine mammary cells are in G_0G_1 (growth) phases.

The data presented here argue for a mammary OAT that has enzymatic properties different from those published for rat liver and kidney OAT. To summarize the data, the bovine mammary enzyme occurs in both cytosolic and mitochondrial fractions, but, because of the similarity in distribution with citrate synthase, these are probably the same enzyme. However, differences in kinetic and thermal properties could either be related to occurrence of true isozymes or to artifacts arising during preparation such as proteolysis. The bovine mammary gland OAT from mitochondria differs from both rat enzymes in pH maximum, in the energy of activation for inactivation, in heat stability, and in K_m. Interestingly, rat kidney and liver and the two bovine mammary enzymes show different behavior when preincubated at 37°C in the absence of substrates. Although the curves for rat liver and bovine mammary gland cytosol are similar in shape (Figure 3), their computed half-lives are different (Table 4). There are apparently no previous reports of OAT in bovine mammary gland and only one previous report of OAT in rat mammary homogenates (18) found in conjunction with a study on arginase.

Comparison of the specific activity of OAT with other bovine mitochondrial enzymes (6, 17) indicates a level lower than for citrate synthase but equivalent to aconitase, $NADP^+$: isocitrate dehydrogenase, and succinate dehydrogenase [54, 28, and 89 nmol/min per mg (6, 17)]. The specific activity of OAT normalized to enzyme lost to cytosol could be somewhat higher.

For metabolic potential (micromoles per minute per gram of tissue at 37°C), a value of .96 can be calculated. This value is low relative to many major bovine mammary enzymes (6, 17). Thus, OAT may need to be considered in the overall scheme of casein synthesis, which can be estimated as follows: using the

starting assumptions of Waghorn and Baldwin (17), a moderately productive animal with a 21.7-kg udder could easily yield 15 kg of milk/d. Based on mean casein content (3), yield is 433 g/d of casein, or 18.8 mmol/d of protein, or roughly 3760 mmol/d of amino acids incorporated into casein protein. Because casein contains 12.3 mol of proline/100 mol of amino acids (H. M. Farrell and H. J. Dower, 1994, unpublished data), 460 mmol of proline/d are incorporated into protein, or 320 μmol/min and 15 nmol/min per g of tissue. This value compares well with the mean proline incorporation of 21.4 nmol/min per g of tissue that was calculated from the overall data of Cant et al. (2). If OAT were to function near maximum velocity, there is a potential to produce 950 nmol/min per g of tissue of L-Δ^1-pyrroline-5-carboxylate, the immediate precursor of proline. However, because K_m for the mitochondrial enzyme is high (8.4 m*M*), a question of enzyme efficiency arises because the mitochondria would require high pools of ornithine to achieve maximum velocity conditions.

CONCLUSIONS

A primary concern of American people today is the need to reduce fat and cholesterol in their diets. During the last few years, the consumption of full fat dairy products has declined. The future goal of the milk producers is to increase protein production while reducing fat production. A better understanding of the mechanisms that control milk protein synthesis and secretion, coupled with an assessment of rate-limiting steps in milk protein production, could point the way to sites of future intervention to alter the protein:fat ratio. This work reports the occurrence of OAT in lactating mammary glands of rat and cow, compares the properties of the crude enzyme preparations to the isozymes of OAT in liver and kidney, and shows that, although not present at limiting concentrations in mammary gland, the K_m is such that OAT efficiency needs to be considered in estimating barriers to protein secretion.

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